Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Fakulteten för veterinärmedicin och husdjursvetenskap
Institutionen för kliniska vetenskaper

What's in the milk?

Aflatoxin and antibiotic residues in cow's milk in Assam, Northeast India.

Hanna Lundén

Uppsala
2015

Degree Project 30 credits within the Veterinary Medicine Programme

ISSN 1652-8697
Examensarbete 2015:64

What’s in the milk?

Aflatoxin and antibiotic residues in cow’s milk in Assam, Northeast India.

Vad finns i mjölken?

Aflatoxin och antibiotikarester i komjölk i Assam, nordöstra Indien.

Hanna Lundén

Supervisor: Johanna Lindahl, institutionen för kliniska vetenskaper, SLU, & International Livestock Research Institute

Assistant supervisor: Ram Pratim Deka, International Livestock Research Institute

Examiner: Ann-Sofi Bergqvist, institutionen för kliniska vetenskaper, SLU

Degree project in veterinary medicine

Credits: 30 hp
Level: Avancerad nivå, A2E
Course code: EX0736

Place of publication: Uppsala
Year of publication: 2015
Number of part of series: Examensarbete 2015:64
ISSN: 1652-8697
Online publication: http://stud.epsilon.slu.se

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Fakulteten för veterinärmedicin och husdjursvetenskap
Institutionen för kliniska vetenskaper

SUMMARY

Worldwide, there are increasing concerns about food safety and what is really present in the food we are eating, and feeding our children. Antibiotic residues are one of these concerns and a growing global problem. In 2001 India became the world's largest consumer of antibiotics and the problem with antibiotic resistance is increasing. Studies have shown a link between a high SCC (somatic cell count) in cow's milk and high risk for antibiotic residues. In India reports have shown that the average number of clinical mastitis is between 3.94% and 23.25% of the total cow population. In additions to antibiotic residues, there is a risk of mycotoxins in milk products. In countries with a tropical climate such as India, problems due to chronic exposure of aflatoxins are emerging. The fungi that produce the toxin, *Aspergillus* spp., thrive in a hot and humid climate. The effects of chronic aflatoxin exposure include liver carcinoma, and potentially immune suppression and stunting, with children being the most susceptible.

In 2009 the International Livestock Research Institute (ILRI) started a project together with local partners to enhance the local informal milk sector in Guwahati, Assam, northeast India. The project involved a training program for milk producers and milk vendors. The project finished in 2013. The objective of this study is to make a follow up on as well as an evaluation of the ILRI project. Trained and untrained farmers were interviewed from a questionnaire, in order to assess the knowledge, attitudes and practices. In addition, milk samples were selected from 25% of the lactating cows on the farms. The milk samples were tested to examine the level of antibiotic residues and aflatoxin in the milk. Of the farmers who participated in this study 74 had received previous hygiene training by ILRI and 76 had not. Both trained and untrained farmers had in average 12 lactating cows. In both trained and untrained farms most of the feeding and caring of the cows was done by the farmer and his/her family members. The overall knowledge about zoonotic pathogens and toxic substances was low. Farmers who had received previous hygiene training by ILRI showed significant higher levels of knowledge (p-value <0.001) than farmers who had not. This was tested by summarizing the results on a number of questions regarding the farmers' knowledge about zoonotic pathogens, toxins and general food safety. The trained farmers had a mean test score of 9.95 and the untrained 8.16.

A rapid testing platform, Charm EZ, was used to test milk samples for residues of Neomycin and Streptomycin, Sulphonamides, β-lactams, Quinolones, Chloramphenicol, Macrolides and Gentamicin, Tetracycline. 88.6% of the collected milk samples tested positive for Neomycin and Streptomycin, the second most common was Sulphonamides that was found in 22.8% of the samples. The results were not significantly different between the trained and untrained group.

The same system was used to test for aflatoxins. 4.5% of the tested milk samples showed a positive result for aflatoxin. The level for a positive result was 500ng/kg which is the limit stated by the U.S. food and drug administration (FDA), Codex Allimentarius and the food safety and standards authority of India (FSSAI). The trained farms had statistically significant more positive samples than the untrained ones (p-value 0.03), which may be explained by the slightly higher milk yield in the trained farms and the presumably higher use of concentrate in the cows' feed.

It is suggested that hygiene training as well as education is implemented to raise the overall knowledge about zoonotic pathogens, toxins and general food safety.

SAMMANFATTNING

Världen över blir problem med livsmedelssäkerhet, och vad som faktiskt finns i maten vi äter och ger till våra barn, en allt större angelägenhet. Antibiotikarester i mjölk och mjölkprodukter är ett av dessa problem som ökar i många länder. 2001 blev Indien den största antibiotikakonsumenten i världen och problem med antibiotikaresistens ökar. Studier har visat ett samband mellan höga celltal i mjölken och antibiotikarester och från Indien finns rapporter om en klinisk mastitförekomst mellan 3.94% and 23.25%. Även mögelgifter är något som kan förekomma i mjölk, och orsakar mer och mer oro. I länder med tropiskt klimat, såsom Indien, ökar följdproblem av kronisk exponering för aflatoxin. Mögelsvampen som producerar toxinet, *Aspergillus* spp., frodas i varmt och fuktigt klimat. Effekter av kronisk exponering för aflatoxin innefattar levercancer, immunosuppression och tillväxtrubbningar. Faran med exponering är särskilt stor för barn.

2009 startade ILRI (The International Livestock Research Institute) ett projekt i staten Assam i nordöstra Indien. Tillsammans med lokala partners ville man förbättra den informella mjölksektorn i och omkring Guwahati, den största staden i regionen. Projektet bestod av två hygienträningsprogram, ett för mjölkproducenter och ett för mjölkförsäljare. Projektet avslutades 2013. Målet med studien var att följa upp och utvärdera effekterna av ILRI:s projekt. Tränade och otränade bönder intervjuades och fick svara på en enkät bestående av 35 frågor. Mjölkprov samlades också in från 25% av de lakterande korna på varje gård. Mjölkproverna analyserades för antibiotikarester och aflatoxin. Av de bönder som svarade på enkäten hade 74 fått hygienträning, medan 76 inte hade fått någon tidigare träning. Både tränade och otränade gårdar hade i medeltal 12 lakterande kor. Större delen av utfodring och skötsel av korna sköttes på alla gårdar i studien av bonden och hans/hennes familjemedlemmar. Den övergripande kunskapen om möjliga zoonotiska patogener och toxiska substanser i mjölk var låg. Bönder som tidigare fått hygienträning hade dock statistiskt signifikant bättre kunskap än bönder som inte fått någon träning (p-värde <0,001). Detta testades genom att summera svaren på ett antal frågor som testade böndernas kunskap om zoonotiska patogener, toxiner och livsmedelssäkerhet i allmänhet. De tränade böndernas medelresultat var 9,95, de otränade böndernas 8,16.

Ett snabbtest, Charm EZ, användes för att testa mjölkproverna för rester av neomycin och streptomycin, sulfonamider, β-laktamantibiotika, quinoloner, kloramfenikol, makrolider och gentamicin samt för tetracyclin. 88.6% av de insamlade mjölkproverna testade positivt för neomycin och streptomycin, den andra mest förekommande antibiotikaresten var sulfonamider som fanns i 22.8% av proverna. Ingen signifikant skillnad i resultat fanns mellan den tränade och den otränade gruppen.

Samma system användes för att testa proverna för aflatoxin. 4,5% av de testade mjölkproverna testade positivt för aflatoxin. Nivån som krävdes för positivt resultat var 500ng/kg vilket är den gräns som satts av the U.S. food and drug administration (FDA), Codex Allimentarius och the food safety and standards authority of India (FSSAI). De tränade gårdarna hade signifikant fler positiva prover än de otränade (p-värde 0,03). Detta skulle kunna förklaras av den något högre mjölkproduktionen på de tränade gårdarna men även att de korna på de tränade gårdarna får en förmodat högre kraftfodergiva.

Det föreslås att hygienträning såväl som utbildning behövs för att öka den allmänna kunskapen om zoonotiska patogener, toxiner och livsmedelssäkerhet i allmänhet.

TABLE OF CONTENTS

INTRODUCTION

Every year, all over the world diseases are caused by food-borne pathogens. The risk is especially high in low-income countries and each year up to 1.4 million children die of diarrhoea. Out of these cases up to 90% of these can be linked to food (WHO, 2004), and animal-source food especially is a risk. However, in milk there may not only be pathogens, there may also be chemicals posing potential hazards to human health. Milk is one of the most important foods worldwide, but also a potential transmitter of zoonotic disease as well as chemical hazards (Fernades *et al*., 2013). Milk-borne pathogens include brucellosis, tuberculosis, listeriosis, salmonellosis, and diphtheria, all diseases that circulate in India today. India is the fourth largest economy in the world today. It has a population of about 1.2 billion people of which, according to the UN, more than a third is living in poverty. It is a diverse country with a mix of modern mega-cities as well as small rural farm villages. It is estimated that more than half of the population is engaged in agriculture and many people depend on animal husbandry for their livelihood (MDG India report, 2014).

In Assam, a Northeastern province in India with 31 million inhabitants, agriculture is an important part of the economy. Agriculture provides employment to more than 50% of the rural population and contributes nearly 25% of the state domestic product. Many of the farmers in Assam are small holders for whom dairy is of great importance. In addition to this, there is a system of vendors and traders, who make their living trading milk, and there are shops, sweet makers, that live on selling sweet milk products. Thus the whole informal dairy sector is important for the livelihood of many people, but there have been concerns about the safety and quality of the milk in Assam (Kumar & Staal, 2010). Therefore, an intervention was started in 2009 to enhance the traditional dairy sector through providing hygiene training and incentive to improve milk quality. Together with its local partners ILRI created two sets of training manuals, one for dairy producers and one for milk traders. Some of the overall objectives with the training were to improve the hygiene and quality of milk produced, to reduce the risk of zoonotic and milk borne diseases and to increase self-esteem, self-satisfaction and social status of the informal dairy market actors. The training was not targeted at antibiotic or afltoxin residues in milk but was mainly concerned about microbial contamination and the effect that it may cause to human health (ILRI, 2013).

Chemical hazards

Antibiotic residues

As a consequence of an unregulated antibiotic market, frequent cases of mastitis and overall poor animal health, antibiotics may be overused. This creates an increased risk of development of resistant bacteria and due to lack of time of restraint of the treated animal's milk, antibiotic residues, as well as other drugs may be present in the milk and other animal products. In developed countries antibiotic use is therefore heavily regulated, and the EU through Regulation (EC) No 396/2005 has established maximum residue limits (MRL) to ensure the lowest possible consumer exposure. In India, as in many other developing countries, antibiotic use is less regulated. How much and which kinds of antibiotics are being used in the region has not been fully investigated, and neither is it known if the producers respect a withdrawal period and if there are any residues in the milk. Although antibiotic residues are a major concern in the developed world, the relative importance of it for human health is not fully elucidated (Stolker & Brinkman, 2005).

Aflatoxins

Mycotoxins, especially aflatoxins, are other chemical hazards in the milk. Aflatoxins are toxins produced in food and feed crops by the fungi *Aspergillus flavus* and *A. parasiticus*. *Aspergillus flavus* thrives in a hot, humid climate and the toxins are of concern due to its impact on human and animal health. One of these toxins produced is aflatoxin B1. Aflatoxin B1 is the most potent naturally occurring chemical liver carcinogen known. It can cause acute liver damage and induces hepatocellular carcinoma (HCC), a form of liver cancer. Acute aflatoxicosis is caused by extremely high doses of aflatoxin and can lead to haemorrhage, acute liver damage and death. HCC can be caused by chronic aflatoxin exposure. Exposure to aflatoxin has also been associated with immune system disorders and diminished weight and height in children, so called stunting. Studies have shown that aflatoxin may have immunosuppressive impacts. It has been suggested that aflatoxin is responsible for the stunted growth in children due to its apparent dose–response relationship with exposure. Aflatoxin also causes liver damage and immunosuppression in animals and reduces milk production in cattle. Aflatoxin M1 is a metabolite produced in the liver when humans or animals consume aflatoxin B_1 and is excreted in the milk. It is similar to aflatoxin B_1 in toxicity and almost as high in carcinogenicity (IARC, 2012).

Objectives

The objective of this study is to investigate the prevalence of aflatoxin and a number of antibiotic residues in milk from trained and untrained dairy farms around Guwahati, Assam, as well as to investigate the knowledge about diseases, biological and chemical hazards among the farmers, as well as their practices regarding treatments of sick cows, feeding practices and food/feed-storage hygiene.

LITERATURE REVIEW

Dairy sector in India

The dairy sector is an important source of income for people in many developing countries. It is not only of economic importance for the 75% of the Indian population that lives in rural areas, of which 38% are poor, but also gives people a source of protein and calcium which in a country with a large vegetarian population like India is of great importance. The milk consumption in India is increasing by six to eight per cent annually and it is one of the worlds top consuming countries (USDA 2012, Douphrate *et al.*, 2013).

In 2001, India, closely followed by the USA, became the world's leading milk producer. Every year Indian cows and buffaloes produce 110 million tons of milk, which is 17% of the world's total milk production. The buffaloes accounted for more than half of that production (FAO, 2010, Douphrate *et al.*, 2013). There are about three times as many dairy producing animals in India as in the USA; the total amount is 38.5 million dairy cows, and approximately 70 million Indian dairy producers (Douphrate *et al.*, 2013). The typical Indian farm is based in the rural parts of the country and family members mostly run the farm business. Over 80% of the cows live in herds of about two to eight animals and yield about one tenth of the milk yield of a typical cow in the USA (FAO, 2010, Douphrate *et al.*, 2013, Hemme *et al.*, 2003). In Assam this remains true; about 82% of the rural households keep cattle and bovine dairy farming is a valuable part of the mixed farming system. The milk production in the state is far below the Indian average and productivity is thus very low. Despite efforts, the organized milk market in Assam remains quite insignificant. Only about three per cent of the milk goes through the formal pasteurized milk and dairy product channels (Kumar & Staal, 2010).

Antibiotic residues in milk

Unpasteurised milk is an excellent mean of transmission of zoonotic and other pathogens to humans. During milking, the milk can easily be contaminated by faeces. In addition, milk is also a great growth medium for microorganisms. The amount of bacteria in the milk gives an indication of the level of milking hygiene, the storage temperature, as well as the time lapsed since milking. The total amount of bacteria indicates the time lapsed since milking, coliform bacteria mostly indicate the level of faecal contamination. International regulations state that milk shall be delivered and refrigerated within 2 h or 3 h after milking (Addo *et al.*, 2011). Today most people purchasing raw milk boil their milk before drinking it. This is a practice that removes most of the bacteria but many of the chemicals that may be contaminating the milk remains due to their often heat stable nature.

There have been reports of milk containing antibiotics and antimicrobial substances in both developing and high-income countries and many studies have shown that there is a clear association between a high milk SCC (somatic cell count) and a high incidence of antibiotic residues, toxins and other pathogenic organisms (Oliver *et al.,* 2005). Indirectly this could indicate that milk with high SCC is associated with health risks to the consumer. Milk with high SCC may also result in dairy products with reduced shelf life and has overall decreased manufacturing properties (Ma *et al.,* 2000). In India, nation-wide reports have shown an incidence of clinical mastitis that varies from 3.94% to 23.25%, and for subclinical mastitis from 15.8% to 81.6% (Joshi & Gokale, 2006). This high prevalence of mastitis indicates both a high SCC but also indicates frequent treatment of cows that may result in antibiotic residues in the milk.

Antibiotic residues, i.e. the remnants and metabolites after antibiotic administration of different antibiotics, such as sulphonamides, beta lactams, tetracycline, may be present in milk and dairy products. Many kinds of antibiotics are used in high dosage when treating diseases in dairy cattle, which increases the risk of residues in the milk. Milk containing antibiotic residues increases the risk of development of antibiotic resistant bacteria in people (Tolentino *et al.,* 2005, Gao *et al.,* 2012) whereas, in allergic people they can cause an allergic reaction, triggered by the antibiotic residues (Stolker & Brinkman, 2005). These are both effects that make dairy products a potential risk to humans. Furthermore the presence of antibiotic residues in milk causes losses in the industry in the processing of yogurt, butter and cheese. This is caused by inhibition of lactic bacteria activity, which makes it not only a public health issue but also an economic necessity to monitor antibiotic residues in milk (Knappstein *et al.,* 2003, Ilic *et al*., 2012).

The major cause of antibiotic residues in the milk is animal treatment with antibiotics. Antibiotic substances can be excreted even after finalized treatment, which is the reason why there in many countries exist established time limits to when the milk is not to be used for human consumption. This time may vary according to national laws and recommendations of the manufacturer (Knappstein *et al.*, 2003). Other potential ways antibiotic residues could end up excreted in milk is if the cow would consume it through the feed or the drinking water.

Depending on the type of samples and the analyses desired, different types of methods has to be used. Today more than 80% of the techniques used to analyse samples for veterinary drugs are based on liquid chromatography–mass spectrometry (LC-MS, or alternatively high performance, HPLC-MS). This is a chemistry technique which uses the combination of the LC capabilities to physically separate compounds with the MS capability of mass analysis. It is a technique with high sensitivity and selectivity (Stolker & Brinkman, 2005). However, these methods require advanced laboratory skills and equipment, and they are quite time consuming, which is why there has also been other more rapid and user-friendly tests developed. One example of these methods is the system used by the Charm EZ reader (CHARM Sciences, 2015), which uses a lateral flow test (LFT), or immunochromatographic test strip technology. This technique uses prefabricated strips made of a material containing dry reagents able to carry solutions. Applying a liquid sample activates the strip reagents. The LFT has great advantages compared to the conventional methods in terms of rapidity and simplicity (Ngom *et al*., 2010, Posthuma-Trumpie *et al.,* 2008). After inoculation of the test strip the system is automatic which makes it possible to operate without previous laboratory skills. The tests take between one and eight minutes (CHARM Sciences, 2015).

Aflatoxin in milk

Moulds, for example *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nominus*, produce aflatoxins, a kind of mycotoxin. Especially in tropical regions these moulds are natural contaminants of feeds of plant origin, especially maize and cereals but also in fruits and nuts (Binder *et al.*, 2007). There are several subtypes of aflatoxin. Four of them may contaminate crops: B_1, B_2, G_1, and G_2 where B_1 is the most potent toxin. In the liver, aflatoxin B_1 (AFB1) is transformed to aflatoxin M_1 (AFM1), which is the main derivate of AFB1, through enzymatic hydroxylation by cytochrome P450-associated enzymes (Jafarian-Dehkordi & Pourradi, 2013). AFM1 is subsequently excreted in milk and urine.

Aflatoxins are potent toxins. In toxicological studies they have been shown to be highly toxic, mutagenic, teratogenic, immunosuppressive and carcinogenic (Jafarian-Dehkordi & Pourradi, 2013). The International Agency for Research of Cancer (IARC) has classified AFB1 as class 1A (carcinogenic to humans) carcinogens. AFM1 have been shown to be approximately 10 times less carcinogenic than AFB1 in rodent experiments but have been classified as class 2B (possibly carcinogenic to humans) by the IARC. The IARC have also identified aflatoxins as one of the most important etiological factors in the development of hepatocellular carcinoma. Calculations show that about 27% of the cases of hepatocellular carcinoma in Southeast Asia likely are induced by aflatoxin (Liu & Wu, 2010). Furthermore, more recent studies have shown a link between childhood exposure of aflatoxin and growth stunting (Gong *et al.*, 2004). Which of the aflatoxins that would be more toxigenic in terms of causing stunting effects in children has not been shown, although AFB1 has been mostly studied and incriminated (Khlangwiset *et al.,* 2011).

Animals who have eaten food or feed contaminated by a high concentration of aflatoxin have suffered from teratogenesis, especially during the first embryonic phase when reabsorption and malformation of foetuses occurred (Scaglioni et al., 2014). So far no studies have shown the same effect in humans.

Studies have shown a direct correlation between milk yield and the carry-over of aflatoxin B1 in the feed to M1 in the milk, where cows with a milk yield of <30kg/day had a carry-over of 1-2% and high-yielding cows (>30kg milk/day) had a carry-over rate of up to 6% (Britzi *et al*., 2013). There have also been studies that show a correlation between the carry-over rate and days in lactation where cows in early lactation (two to four weeks after calving) a higher carry-over rate than cows in late lactation (34 to 36 weeks after calving) (Veldman *et al*., 1992).

Aflatoxins are in general stable during heat-treatments. Studies have shown a certain reduction of the concentration but there have been great differences in analytical methods, wide range of temperatures and use of both naturally and artificially contaminated milk (Siddappa *et al.,* 2012).

In a study of 52 samples of raw and pasteurized milk from Karnataka and Tamilnadu 61.6% were positive for aflatoxin M1 and 17.3% of them had levels over 0.5μg/kg. In the same study plain UHT milk was analyzed and all samples where positive, with 38% having levels over 0.5μg /kg (Siddappa *et al.,* 2012). There have been other Indian reports that have discovered prevalence of Aflatoxin M1, for example in raw and pasteurized milk in 94% samples collected in Gujarat in the western part of India. The concentration in the samples ranged between 0.066 and 0.763μg/kg (Choudhary *et al*., 1997). In a third study made in 1995 by Rajan *et al.* (1995) found 17.7% contaminated samples when they analysed

504 raw milk samples from Kerala where the concentration were 0.1–3.5µg /kg. In 1976 Paul *et al*. analysed raw milk samples from Ludhiana in the north of India and found 6.2% positive samples.

In order to minimize the potential risk, the World Health Organization (WHO) has recommended that the level of AFM1 should be reduced. The limit set by the European Commission for AFM1 in raw milk, treated milk and dairy products is 50ng/kg. The amount of AFM1 is even recommended not to exceed 25ng/kg for infant milk, infant formulae and special food products (Jafarian-Dehkordi & Pourradi, 2013). However, limits vary between countries, and the U.S. food and drug administrstion (the FDA) recommends 500 ng/kg (FDA, 2005), which is also the limit prescribed by the Codex Alimentarius Commission (2001) and by the obligatory protocols in India, the food safety and standards authority of India (FSSAI) Regulations (2011).

Much of the cattle feed in India have been found to have a great content of aflatoxin. In 1995 Dhawan and Choudhary conducted a study that showed an average of 103µg/kg (range 3-1734µg/kg) in cattle feedstuffs. In India maize and peanut cake is a big part of livestock feed. Levels of AFB1 as high as 3300µg/kg have been reported in these ingredients (Siddappa *et al.*, 2012). If a cow shall produce milk with AFM1 less than 0.05µg/kg, recommended by WHO, Veldman *et al.* (1992) estimated that the individual intake should be less than 40µg per cow and day.

MATERIAL AND METHOD

Location

The study was made as a minor field study (MFS) funded in part by Sida, the Swedish international development cooperation agency. The fieldwork was conducted from September 1st to November 15th 2014 around the city of Guwahati, capital of the state of Assam in Northeast India. Situated between the banks of Brahmaputra and the foothills of the Shillong range, the state hosts a great variety of rare wild animals such as Asian elephants, Bengal tigers and one horned rhinoceros. The climate is humid subtropical with a rain period May – September with rainfalls up to 340mm. During September to November, the average low is 21.1 degrees and the average high is 29.7 degrees and the average rainfall during this period is around 100 mm per month (National Oceanic and Atmospheric Administration). The study was conducted during the end of the rain period.

Survey

From a sampling frame of all trained and untrained farmers in Guwahati, Assam, 100 were randomly selected in each category. There was already a sampling frame for all trained producers, since they had been registered for training. A sampling frame of untrained farmers was created by the lead village farmer that acted as the local guide. Since there was a risk that some farmers could not be identified using the sampling frames, or would not be willing to participate in the study, the next farmer on the list was selected as a replacement. The farmers were informed about the purpose of the study and asked for willingness to participate. If verbal or written consent was given, local enumerators, trained on using the questionnaires, interviewed the farmers.

Data was collected on the numbers of cows at the farm, the milk yield, disease problems experienced, common treatments and feeding practices. The tools, the study design and the consent procedures had undergone an ethics approval procedure at International Livestock Research Institute before the study commenced (IREC 2014-08). During the interviews the farm was examined as to hygiene and feed storage practices. The animals were also individually examined and milk sampled. The number of cows sampled was based on the number of milking cows at the farm and was set to a minimum of 25% with a minimum of three cows selected.

Due to restraints in the field all selected farms could not be visited. A total of 150 farms, 74 trained and 76 untrained in five regions around Guwahati were visited. This study focuses on the incidence of aflatoxin and antibiotic residues in the milk and the general food safety awareness. The remaining data is presented in two different reports (Berg 2015, Melin 2015)

Milk sampling

Milk and data collection from farms were acquired from September 15th to November 1th, 2014. The animals were chosen according to randomization; for example, if the farm had 20 lactating cows, every fourth lactating cow were chosen for examination, with varying start numbers. Pooled samples from all four udder quarters were collected in sterile tubes and transported to the lab in a cooling box. For the aflatoxin and antibiotic residue testing the samples were frozen until analyses.

Analyses of aflatoxin and antibiotic residues were done using a Charm EZ platform (Charm Sciences, MA, USA) that uses a LFT technique. The milk samples from each cow were tested for aflatoxin. After being tested for aflatoxin all samples from one farm were pooled and tested for antibiotic residues (β-lactams, Tetracycline, Quinolones, Sulphonamides, Gentamicin and Macrolides, Neomycin and Streptomycin, Chloramphenicol).

Statistical analysis

Based on the positive and negative results, prevalence for aflatoxin was calculated on cow level and antibiotic residues were calculated on farm level. Positive and negative results were tabulated by descriptive statistics and analysed with a χ^2–test in order to see if there exists a significant difference between trained and untrained farmers. Milk production was compared with descriptive statistics and with a two sample unpaired t-test in order to see if there existed a significant difference. The answer that showed knowledge about the questions was given one point and the wrong zero. Maximal test score was 18. The farmers’ knowledge score about milk hygiene, diseases and hygiene practises was then tested with a two-sample t-test in STATA.

RESULTS

Demographics

Of the farmers who participated in this study 74 had received previous hygiene training by ILRI and 76 had not. The trained respondents were 95% (n=70) male and the untrained 96% (n=73) male. Both trained and untrained farmers had in average 12 lactating cows. The trained farms ranged between 3 and 35 lactating cows with a median of 15 cows. In the untrained farms the number of lactating cows was between 2 and 46 with a median of 12 cows.

In the trained farms, 46 % of the respondents did the feeding themselves. 19% took help from someone else in the family (wife, son, brother or father). In 16% of the cases unspecified family members did the feeding. In the untrained farms 57% of the respondents did the feeding themselves. In 14% of the farms they had help from various family members (see table 1).

Table 1. Person in the household responsible for feeding dairy cows in Assam, India

Who in the household is responsible for feeding?	trained	untrained
Self	34	42
Self + labour	7	6
Self + wife	4	4
Self + wife + son	1	0
Self + son	3	4
Self + father	1	0
Self + brother	1	0
Self + father + brother	1	0
Self + family members	4	4
Wife	0	3
Son	4	2
Family members	12	9
Labour	2	2

Diseases and milk yield

The most common diseases reported by the farmers were mastitis (12.8%), fertility problems (8.7%), laminitis (7.4%) and inappetence or emaciation (6.0%). Before hygiene training there was no statistical significant difference in milk yield/day. After training there was a significant difference (p-value 0.003) of 0.95 litres higher per cow/day (see table 2).

Table 2. Average milk production in trained and untrained farms in Assam, India, before the training/two years ago and today.

	Average milk production in liters per cow and day 2 years ago/before ILRI training	Average milk production in liters per cow and day now
Trained farmers	7.0 (range 2.5-10)	7.8 (range 3-15)
Untrained farmers	7.3 (range 2.5-14)	6.8 (range 2.5-14)

When asked, the trained farmers thought their cows were healthier than before hygiene training. 87 % (63/72) said they were healthier and the remaining nine farmers that answered the question didn’t see any difference in health status. Out of the untrained farms, 11 thought their cows were less healthy than two years ago and 52 did not see any difference. Four of the untrained respondents thought that the cows were healthier than two years ago.

Antibiotics and treatment

In the trained households 82% (n=61) of the respondents decided themselves when a sick animal should be treated with medicine. Two of the four female respondents did decide themselves, one together with her husband and in the last case the husband decided. The person responsible for deciding if a sick animal should be treated with medicine was in 88% (n=67) of the untrained farms the respondent. In the untrained farms, one of the female respondents made decisions about treatment herself, one with help from her brother, in one household the husband made the decision. Two of the male respondents said their wife made the decisions.

Table 3. Person responsible for deciding if a sick dairy cow shall be treated with medicine.

Who in the household is responsible for deciding if a sick animal shall be treated with medicine?	**Trained**	**Untrained**
Self	61	67
Self + husband	1	0
Self + wife	1	1
Self + labour	1	0
Self + father	1	0
Self + brother	1	1
Self + son	1	2
Son	3	0
Father	1	0
Husband	1	1
Wife	0	2
Family members	2	2

Most of the farmers took advice from a veterinarian what antibiotics to use (table 4). Only four of the interviewed farmers gave a response on how long they kept an antibiotics vial. Of them, three (trained) respondents said they used an open antibiotic vial at once. The last one (untrained) said he did not store antibiotics himself.

Table 4. Advisors on antibiotic use for dairy cows in Assam, India

Who advice you what antibiotics to buy and use?	**Trained (n=74)**	**Untrained (n=76)**
Veterinarian	66	70
Vet. field assistant	22	22
Neighbour	2	1
Self	10	5
Pharmacist	1	1
Other	0	0

25.6% of the trained farmers agreed with the statement ”toxic substances can be present in milk” in the untrained group only 7.8% agreed with the statement. In the trained group 35.1% of the farmers agreed with the statement ”toxic substances can cause harm to humans” in the untrained group 13.2% agreed. 28.4% of the trained farmers agreed with the statement ”antibiotic residues from treating the animals can be present in the milk” and 10.5% of the untrained farmers agreed.

Table 4. Knowledge of dairy farmers in Assam, India, regarding hazards in milk

Do you agree or disagree with the following statements?	**Trained (n=74)**		**Untrained (n=76)**	
	Agree	**Disagree/ Uncertain**	**Agree**	**Disagree/ Uncertain**
Drinking milk is good for your health?	73	1	75	1
Safe milk can be judged by sight?	69	5	76	0
Safe milk can be judged by taste?	73	1	76	0
Toxic substances can be present in milk?	19	55	6	70
Toxic substances can cause harm to humans?	26	48	10	66
Antibiotic residues from treating the animals can be present in the milk?	21	53	8	68

66.2% of the trained farmers thought disease from a cow can be spread through drinking raw milk, of the untrained farmers 47.3% answered ”yes”.

Of the trained farmers 56.7% answered ”yes” when asked if disease could spread from cow to human by getting milk on your hands and not wash them, 38.2% of the untrained farmers answered yes.

90.5% of the trained and 76.3% of the untrained farmers thought you could get sick by getting manure from a cow on your hands and not wash them. Of trained farmers, 90.5% answered yes, there is a risk from helping a cow deliver a calf, while in the untrained group 76.3% answered yes. Similarly, 85.1% of trained farmers and 59.2% of untrained farmers knew the risk of disease from getting cow saliva or fluid on you.

Table 5. Knowledge and attitudes towards milk and hygiene among farmers in Assam, India

Do you think disease from a cow can be transmitted in the following ways?	**Trained**		**Untrained**	
	Yes	**No/ uncertain**	**Yes**	**No/ uncertain**
Drinking raw milk?	49	15	36	40
Drinking boiled milk?	0	74	0	76

From getting raw milk on your hands and not washing them?	42	32	29	47
From getting manure on your hands and not washing them?	67	7	58	16
From helping a cow deliver a calf?	67	7	58	16
From getting cow saliva or other fluids on you?	63	11	45	31

Almost all the trained as well as the untrained farmers said they did not use the milk from a cow that was under treatment with antibiotics. 2.7% of the trained and 6.6% of the untrained sold milk from cows treated with antibiotics. 4.0% of the trained and 9.5% of the untrained said they did not throw away the milk. Two of the untrained farmers had not used antibiotics so far.

Table 6 Handling of milk from antibiotic-treated cows in Assam, India

What do you do with the milk, when the cows are under treatment with antibiotics?	Trained		Untrained	
	Yes	**No**	**Yes**	**No**
Own drinking	1	73	1	75
Sell	2	72	5	71
Throw away	71	3	69	7
Mix with other milk	0	74	1	75
Give to calf	0	74	0	76

The amount of antibiotic residue in the milk samples is shown in Table 7. Largest amount of samples were positive for aminoglycosides (neomycin and streptomycin), which was present in 88.6% of the samples. Sulphonamides were the next most common and were present in 22.8% of the samples. β-lactams 2.9%, quinolones 2.0%, chloramphenicol 2.3%, and macrolides and gentamicin 2.0%. One of the tested farms was positive for tetracycline. The results were not significantly different between the trained and untrained group.

Table 7 Antibiotic residues detected in milk from dairy cows in Assam, India

	Trained	Untrained
QUAD2 (macrolides and gentamycin)	1/47	1/55
MRLBL1 (β-lactams)	1/48	2/56
Sulphonamides	11/48	12/53
Tetracycline	1/55	0/45
Chloramphenicol	1/41	1/47
Neomycin and streptomycin	35/42	43/46
Quinolones	2/44	2/55

Only 12 out of 152 responded to what antibiotics they normally used (8 trained, 4 untrained). The most frequent antibiotic amongst the trained farmers was Ceftiaxone, which is a third generation cephalosporin, 5 of the 8 farmers who answered used this. One of the farmers used Ceftriaxone, another cephalosporin, in combination with Tazobactum, a substance that broadens the spectra to include β-lactamase producing bacteria as well. The other antibiotics were Cloxacillin (a broad-spectrum antibiotic in the same class as penicillin), Tetracycline, Oxytetracycline, Dicrystin (a mix of Streptomycin, Procaine and Penicillin G) and Penicillin.

Two of the untrained farmers used Ceftriaxone, one Ceftiofur that is also a third generation cephalosporin. One used Mammitel (a form of cloxacillin) and Pendistrin SH (a combination of Streptomycin, Penicillin and procaine).

Feed storage hygiene and aflatoxins

All the 152 respondents reported that green fodder as well as dry fodder was stored outside, whereas concentrates and feed ingredients were stored inside. There were no differences between the two groups as to what they fed their cows.

Picture 1 Example of dry and green fodder storage

Table 8 Storage of feed in Assam, India

What do you feed your cows?	Trained Yes	No	Untrained Yes	No
Dry straw	74	0	76	0
Green fodder	74	0	76	0
Concentrate	19	55	17	59
Feed ingredient	73	1	73	3

Only seven farmers said their feed got mouldy (five trained, two untrained). Two trained farmers had heard about aflatoxin, none of the untrained farmers. One trained farmer said that aflatoxin could cause disease. Many farmers did not give any answer to what they did do with mouldy feed (table 9).

Table 9 Handling of mouldy cattle feed in dairy farms in Assam, India

What do you do with mouldy feed?	Trained Yes	No	Untrained Yes	No
Feed to cattle	0	36	0	36
Dispose of	9	29	9	28
Sell	0	36	0	36

As shown in table 10, a higher amount (15) of trained farmers thought that moulds are harmful to human health (p-value 0.0002).

Table 10 Knowledge about moulds in dairy farms in Assam, India

Are moulds harmful to human health?	yes	no
trained	15	58
untrained	6	70

The farmers who answered yes to the question in table 10 were asked to specify what they knew about the negative effects of moulds. 11 out of the 15 trained farmers answered. Two said it is harmful, 4 said it causes disease, 3 that it causes dysentery, and 2 that it causes gut problems. Six untrained farmers answered. Three said they thought it caused disease, one that it may cause harm to animals, one that it may cause severe diarrhoea, and one said he did not know.

Only two of the trained farmers said they knew about aflatoxin, none of the untrained had heard about it. One of the trained farmers that had heard about aflatoxins thought it could cause disease, 8 did not answer and the rest answered they did not think it could cause disease (n=64). Out of the trained farmers no one thought aflatoxin could cause disease, 11 gave no answer.

14 out of 310 (4.5%) of the tested cows showed positive results for aflatoxin, where a positive result is a result over 500ng/kg. Two of the cows had a level as high as 750ng/kg, the maximum limit of the test. Of the 137 cows that were not only tested for a positive/negative result but also given a numeric value, 14 tested over 50ng/kg, which is the limit set for milk products in the EU. Four of them had values over 500ng/kg. There was a statistically significant difference between the two groups where the trained group had a higher amount of positive cows (7.2%) than the untrained (2.3%) ($p = 0.03$).

Table 11 Aflatoxin levels detected in milk from dairy cows in Assam, India

Aflatoxin	**Positive (>500ng/kg)**	**Negative (<500ng/kg)**	**Total**
Trained	10	128	138
Untrained	4	168	172
Total	14	296	310

Based on the assumption that as many of the negative results above have a concentration of aflatoxin over 50ppt as in the 137 samples tested for numeric aflatoxin concentration another 20 samples would be positive. That would give a total of 34 positive results and a prevalence of samples with levels above 50 ng/kg of 11%.

The general knowledge of the trained farmers was higher when compared to the untrained ones when the answers to the questions “What do you do with the milk, when the cows are under treatment with antibiotics?”, “Are moulds harmful to animal and human health?”, “Have you heard about aflatoxins?”, “Do you think disease from a cow can be transmitted in the following ways?” where a number of alternatives were given (see table 5) and “Do you agree or disagree with the following statements?” also with a number of statements given (see table 4).

When the knowledge ”test” scores were compared in a two-sample t-test the trained farmers had a significantly ($p<0.001$) higher score; trained farmers had a test score of 9.95 and untrained 8.16.

DISCUSSION

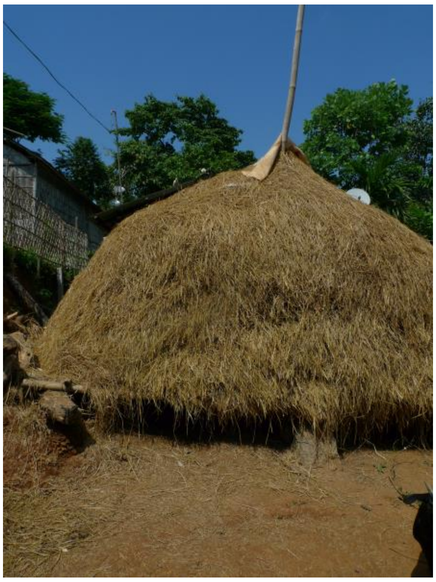
This study focuses on the hidden risks in milk posed by antibiotic residues and aflatoxins, and the knowledge levels of dairy farmers regarding hygiene and risks with milk. The use of antibiotics increases worldwide. This causes problems such as development of resistant bacteria. Between 2000 and 2010 the consumption of antibiotic drugs increased by 36% worldwide. In 2010 India emerged as the world's largest consumer of antibiotics, and together with Brazil, China; Russia and South Africa it accounts for 76% of the global increase. Between 2001 and 2010 India's consumption increased from eight billion units to 12.9 billion units. (Van Boeckel *et al*., 2014). The market is free and you don't need a prescription to buy antibiotics.

This study found a great number of samples that tested positive for the aminoglycosides streptomycin and neomycin. These are two types of antibiotics that in veterinary medicine are used to treat serious infections with gram-negative bacteria. Why we found so many positive samples is unclear. When asked only two of the farmers mentioned that they used a drug containing aminoglycosides but only 12 of 152 farmers answered the question, so conclusions as to what antibiotics is the most common cannot be drawn. It may well be that the treating veterinarian prefers to use medicines containing aminoglycosides; almost none of the farmers stored antibiotics himself and might therefor be unaware of what treatment the veterinarian uses for his cows. The veterinarian may prefer to use some sort of aminoglycoside due to low price relative other antibiotics or due to some kind of advertising campaign. It might also be that the farmers use some kind of salve or mixture that they do not know contains antibiotics. This is speculation and more surveys have to be done on the subject to acquire more knowledge. The fact remains that in India the overall use of aminoglycosides has increased. In 2010 the total use was 10^8 units, which was an increase of almost $5*10^7$ since 2000 (Van Boeckel *et al*., 2014). High-level of aminoglycoside resistance have been detected in various studies. In 140/1000 (14%) consecutive isolates of *Enterobacteriaceae* selected from India showed resistance to aminoglycosides (Hidalgo *et al*., 2013). This gives an indication that the use is quite widespread in human medicine and one can suspect that this is the case in animal medicine as well. Why the use has increased have to be studied further.

The bacteria cultivated from the milk samples were all gram-positive (Melin 2015). The coagulase negative staphylococci were not tested for pencillinase production but all of the *Staphylococcus aureus* tested negative for penicilinase production and were all sensitive to penicillin (Melin 2015). This should be considered when treating mastitis in the region to avoid using antibiotics with an unnecessary broad spectrum.

The cows from the trained farms had a higher amount of aflatoxin in the milk. There were no apparent differences in what they were fed when compared to the cows on the untrained farms. Studies have shown that high yielding cows have a higher carry-over of aflatoxin from blood to milk than low yielding (Veldman *et al*., 2010, Masoero *et al*., 2007). These studies were made on, relative to the Assamese cows, high yielding cows, which produced about 30 litres of milk/day. The cows in this study yielded between two and 15 litres per day and it has not been studied whether there is a difference in carry-over rate when the differences in milk yield are relatively few litres. We did not ask questions about the amount of feed and concentrate the farmers gave their cows in our survey and therefor one cannot draw any conclusions to whether the trained farmers give their cows more concentrate per cow or not. This could have been an explanation to why there are more positive cows in the trained farms. The concentrate contains a high amount of maize, a crop that is very sensitive to *Aspergillus* spp infection, cows that eat and produce more are therefor at risk due to higher intake. In order to evaluate this, further studies looking into the amount of concentrate given and the different feeds consumed by the cows are needed.

4.5% of the cows tested positive for aflatoxin with levels >500ng/kg. This is lower than earlier studies that have shown results between 6.2% and 94%. If we include the calculated amount of samples with concentrations over 50ng/kg the prevalence is 11.0%, which is in line with previous studies. All the 14 samples had a concentration of 500ng/kg or higher which is 10 times higher than the MRL in the EU (50ng/kg), and would also exceed the generous limits of the US. Given the high amount of aflatoxin present in the milk there is reason to believe that lactating cows are sometimes fed mouldy feed. The hay/straw is stored outside in large stacks (see picture 2), in the often very hot and humid climate moulds are given a very good environment to grow. The sub tropical climate is in general well suited for various types of moulds, such as *Aspergillus* spp. A great part of the concentrate consists of maize, which is one of the feeds that are most sensitive to contamination by *Aspergillus* spp. In spite of this, very few farmers said they had problems with their feed getting mouldy. This may indicate that farmers are not recognizing mould for what it is, and more training may be needed. Nine trained and nine untrained farmers said they disposed of mouldy feed. None of the farmers said they gave mouldy feed to the cows or sold it. The question remains to be answered what actually happens with the mouldy feed, if there are any, which there is reason to believe.



Picture 2 Typical storage of dry fodder

One theory could be that farmers are very bad at detecting moulds or classifying it correctly. Since there is a general low awareness that moulds are at all harmful the farmers may not be receptive to seeing it. Maybe a more accurate estimate could have been obtained if there had been pictures in the survey that showed the farmers examples of mouldy feed.

No studies have been made where the Charm EZ have been compared to other analytical instruments. The company states that it has a high sensitivity and specificity, and in addition the β-lactams test is approved by the FDA for use as a farm side test for detecting β-lactams. The LFT is in general considered highly sensitive and able to detect even low concentrations of residues (Ngom *et al*., 2010, Posthuma-Trumpie *et al.*, 2008).

There was a higher general awareness among the trained farmers about toxic substances and that they pose a potential risk to human health. There was also a greater insight in the zoonotic potential of manure, raw milk, and body fluids from the cows. This suggests that the trained farmers have acquired knowledge about these things through the training programs. However, the knowledge was not as high as one could wish. When we scored the answers to the questions in table 4, 5, 6 and 10 together with two other questions the "test" total was 18 points and not one of the farmers scored higher than 14 points and most of them scored below 10 points. This suggests that there is a hole in the knowledge base that needs to be filled. The difference in test scores between trained and untrained farmers was statistically significant but there is still the possibility that the farmers who volunteered for the training program was the ones who were already more active in seeking new knowledge to begin with.

The major part of the farmers who took part in the survey managed the feeding of their animals themselves and in those cases where there were other people involved it was usually members of the family who helped. The same were true for who decided when an animal should be treated. Most of the farmers made these decisions by themselves and in case others helped it was in most cases family members. This points out that the farm owner and their family is a key to making a difference in how sick animals are handled and how the milk and feed is treated. By spreading knowledge not only about diseases and their zoonotic potential, but also about how raw milk should and should not be handled, quite a change in attitudes could be achieved. If teaching the farmers about moulds could create greater awareness about the toxic effects and potential danger of feeding your cows mouldy feed the prevalence of aflatoxin could be lowered. Almost none of the farmers had heard of aflatoxin and most did not know that moulds were harmful. This suggests that animals probably is fed mouldy feed and also that the farmers and their families are put at risk when handling mouldy feed.

When making training interventions one of the concerns is that if the farmers do not see the benefits from it, the new knowledge may not be put in practice. If no benefits can be shown to public health or to the economy, decision makers may be unwilling to maintain a training system. In this study we can see that training has an effect not only on the farmers' economy and the health of the animals, but also on the general knowledge. However, there are still gaps. The knowledge about biological and chemical hazards could be much higher and one could make differences to both the farmers' and the public's health by continuing the training especially if information about moulds and aflatoxins should be added to the list of information.

CONCLUSION

This study shows the impact of training on general knowledge of farmers, but not on the chemical risks in milk. The amount of aminoglycosides was surprisingly high in the collected samples, and although no previous studies have examined the prevalence of aminoglycosides, there are studies that show that the use increases and also the resistance. This is a cause for concern and further studies are needed to investigate why the level is so high and if this is the case in the rest of the country. The prevalence of milk with aflatoxin samples over the limit are slightly lower than previous studies but this is the first study made in the Northeastern part of India. The levels of aflatoxin found is high and therefore pose a threat to humans, especially children, why this should be studied further and education and information about moulds and their potentially harmful effect on human health should be carried out.

The general knowledge about diseases, biological and chemical hazards were low. The farmers who had received training seemed to be slightly better educated but there is still some gaps to fill. This study concludes that training of farmers have effects, although there are still improvements to be made, and the recommendation for the future would be to improve the program and extend it to more farmers.

AKNOWLEDGEMENTS

SIDA for the scholarship, and ILRI for the funding that enabled this study.

My very suporitive supervisor Johanna Lindahl.

Ram Pratim Deka, my supervisor in India.

Anhil, Anop, Gunajit and Dr. Bora for much needed help in the field.

Anna Berg and David Melin for making this field trip a memory for life.

REFERENCES

Addo, K.K., Mensah, G.I., Aning, K.G., Nartey, N., Nipah, G.K., Bonsu, C., Akyeh, M.L. & Smits, H.L. (2011). Microbiological quality and antibiotic residue in informally marketed raw cow milk within the coastal savannah zone of Ghana. *Tropical and International Health*, 16:117-232.

Agarwal, J., Kaylan, R. & Singh, M. (2009). High-level aminoglycoside resistance and B-lactamase production in Enterococci at a tertiary care hospital in India. *Jpn. J. Infect. Dis.* 62:158-159.

Binder, E.M., Tan, L.M., Chin, L.J. & Richard, J. (2007). Worldwide occurrence of mycotoxins in commodities, feed ans feed ingredients. *Anim. Feed Sci.Tech*, 137:265-282.

Britzi, M., Friedman, S., Miron, J., Solomon, R., Cuneah, O., Shimshoni, J.A., Soback, S., Ashkenazi, R., Armer, S. & Shlosberg, A. (2013). Carry-over of Aflatoxin B1 to Aflatoxin M1 in high yielding Israeli cows in mid- and late-lactation. *Toxins*, 5:173-183

Codex Alimentarius Commission. (2001). CODEX STAN 232-2001 Available at:

<http://www.codexalimentarius.org/standards/list-of-standards/en/

?provide=standards&orderField=fullReference&sort=asc&num1=CODEX>.

CHARM Sciences. (2015) http://www.charm.com/fr/instruments/featured-systems/charm-ez.html (2015-01-27)

Choudhary, P.L., Sharma, R.S., Borkhatriya, V.N., Murthi, T.N. & Wadodkar, U.R. (1997). Survey on the levels of aflatoxin M1 in raw and market milk in and around Anand town. *Indian J. Dairy Sci.*, 50:156–158.

Dhawan, A.S. & Choudhary, M.R. (1995). Incidence of aflatoxins in animal feedstuffs: a decade's scenario in India. *J. AOAC Int.*, 78:693–698.

Douphrate, D., Lunner Kolstrup, C., Reynolds, S. J., Jakob, M. & Kinsel, M. (2013). The Dairy Industry: A Brief Description of Production Practices, Trends, and Farm Characteristics Around the World (2013), *Journal of Agromedicine*, 18:3:187-197.

European Commission Regulation. (2001). Setting maximum levels for certain contaminants in food stuffs. 466/2001/EC. pp.1–13. Available at: <http://ec.europa.eu/food/fs/sfp/fcr/fcr02_en.pdf>.

Fernades, S.A. de Albuquerque, Magnavita, A.P.A., Ferrao, S.P.B., Gualberto, S.A., Faleiro, A.S., Figueiredo, A.J. & Matarazzo, S.V. (2014). Daily ingestion of tetracycline residue present in pastureized milk: a public health problem, *Environ Sci Pollut Res.*, 21:3427-3434.

Food Safety and Standards Authority of India (FSSAI). (2011). *Food Safety and Standards (Contaminants, toxins and residues) Regulations.*

<http://www.fssai.gov.in/Portals/0/Pdf/Food%20safety%20and%20standards%20

(con taminats,%20toxins%20and%20residues)%20regulation,%202011.pdf>.

Gao J., Ferreri M., Liu X.Q., Chen L.B., Su J.L. & Han B. (2012). Development of multiplex polymerase chain reaction assay for rapid detection of Staphylococcus aureus and selected antibiotic resistance genes in bovine mastitic milk samples. *Journal of Veterinary Diagnostic Investigation*, 23:894–901.

Gong, Y., Hounsa, A., Egal, S., Turner, P., Sutcliffe, C., Hall, A.E., Cardwell, A.J. & Wild, C.P. (2004). Post weaning exposure to aflatoxin results in impaired child growth: a longitudinal study in Benin. *West Africa. Environ. Health Perspect.*, 112:1334–1338.

Hemme, T., Garcia, O. & Saha, A. (2003). A review of milk production in India with particular emphasis on small scale production. *PPLPI Working Papers*, URL:http://purl.umn.edu/23777 (2014-12-15)

Hemme, T., Mohi Uddin, M. & Asaah Ndambi, O. (2014). Benchmarking Cost of Milk Production in 46 Countries. *254 Journal of Reviews on Global Economics*, 3:254-270

Hidalgo L., Hopkins K.L., Gutierrez B., Ovejero C.M., Shukla S., Douthwaite S., Prasad K.N., Woodford N. & Gonzalez-Zorn B. (2013). Association of the novel aminoglycoside resistance determinant RmtF with NMD carbapenemase in enterobacteriaceae isolated in India and the UK. *J Antimicrob Chemoter*, 68:1543-50

International Livestock Research Institute. (2013). Training on Hygienic Milk Production and Handling. Report of a training course held at Guwahati, Assam, India in 2013. Nairobi, Kenya: ILRI.

International Agency for Research on Cancer. (2012). IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Vol. 100F. IARC.

Ilić K., Jakovljević E. & Skodrić-Trifunović V. (2012). Social-economic factors and irrational antibiotic use as reasonsfor antibiotic resistance of bacteria causing common childhood infections in primary healthcare. *Eur J Pediatr.*, 171:767-77

Jafarian-Dehkordi, A. & Pourradi, N. (2013). Aflatoxin M1 contamination of human breast milk in Isfahan, Iran. *Advanced Biomedical Research*, 2:86.

Joshi, S. & Gokhale, S. (2006). Status of Mastitis as an Emerging Disease in Improved and Periurban Dairy Farms in India. *Ann. N.Y. Acad. Sci.* 1081:74–83

Khlangwiset, P., Shepard, G.S. & Wu, F. (2011). Aflatoxins and growth impairment: A review. *Critical Reviews in Toxicology*, 41:740-755

Knappstein K., Suhren, G. & Walte, H-G. (2003). Influence of milking frequency on withdrawal period after application of B-lactam antibiotic-based drugs. *Analytica Chimica Acta*, 483:241–249

Kumar, A. & Staal, S.J. (2010). Is traditional milk marketing and processing viable and efficient? An empirical evidence from Assam, India. *Quarterly Journal of International Agriculture*, 49:213-225.

Liu, Y. & Wu, F. (2010). Global burden of aflatoxin-induced hepatocellular carcinoma: a risk Assessment. *Environ. Health Perspect.,* 118:818–824.

Ma, Y., Ryan, C., Barbano, D.M., Galton, D.M., Rudan M.A. &Boor K.J. (2000). Effects of somatic cell count on quality and shelf-life of pasteurized fluid milk. *J Dairy Sci*., 83:264-74.

Masoero, F., Gallo, A., Moschini, M., Piva, G. & Diaz, D. (2007). Carryover of aflatoxin from feed to milk in dairy cows with low or high somatic cell counts. *Animal*, 1:9:1344-1350

Medina, A., Rodriguez, A. & Magan, N. (2014). Effects of climate change on Aspergillus flavus and Aflatoxin B1 production. *Front Microbiol*., 22:5:348.

Millennium Development Goals India report 2014. (2014). http://www.in.undp.org/content/dam/india/docs/MDG%20%20India%20Report%202014.pdf (2015-01-27)

Ngom, B., Guo, Y., Wang, X. & Bi, D. (2010). Development and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: a review. *Anal Bioanal Chem*, 397:1113–1135

Oliver S.P., Boor, K.J., Murphy, S.C. & Murinda, S.E. (2009). Food safety hazards associated with consumption of raw milk. *Foodborne pathogens and disease*, 6:7:793-806.

Oliver S.P., Jayarao, B.M. & Almeida, R.A. (2005). Foodborne pathogens in milk and the dairy farm environement: food safety and public health implications. *Foodborne pathogens and disease*, 2:2:115-135,

Paul, R., Kalra, M.S. & Singh, A. (1976). Incidence of aflatoxin in milk and milk products Northern state of Ludhiana Punjab. *Indian J. Dairy Sci.,* 29:318–321.

Rajan, A., Ismail, P.K. & Radhakrishnan, U. (1995). Survey of milk samples for aflatoxin M1 in Thrissur, Kerala. *Indian J. Dairy Sci.,* 48:302–305.

Scaglioni, P.T., Becker-Algeri, T., Drunkler, D. & Badiale-Furlong, E. (2012). Aflatoxin B1 and M1 in milk. *Analytica Chimica Acta*, 829:68-74

Vinutha Siddappa, Nanjegowda, D.K. & Viswanath, P. (2012). Occurrence of aflatoxin M1 in some samples of UHT, raw & pasteurized milk from Indian states of Karnataka and Tamilnadu. *Food and Chemical Toxicology,* 50:4158–4162

Posthuma-Trumpie, G.A., Korf, J. & van Ameronge, A. (2009). Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey. *Anal Bioanal Chem*, 393:569-582

Stolker, A.A.M. & Brinkman, U.A.T. (2005). Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals—a review. *Journal of Chromatography A*, 1067:15-53

Tolentino, R.G., Perez, M.N., Gonzalez, G.D., Arain, M.A., Leon, S.V., Lopez, M.G. & Flores, G.P. (2005). Determination of the presence of 10 antimicrobial residues in Mexican pasteurized milk. *Interciencia,* 30:291–294

USDA Foreign Agricultural service. (2012). GAIN (Global Agricultural Information Network) report IN2132, India: Dairy and Products annual.

US Food and drug administration. (2005). *Whole Milk, Lowfat Milk, Skim Milk - Aflatoxin M1*. http://www.fda.gov/ICECI/ComplianceManuals/CompliancePolicyGuidanceManual/ucm074482.htm (2015-01-27)

Van Boeckel, T.P., Gandra, S., Ashak, A., Caudron, Q., Grenfell, B.T., Levin, S.A. & Laxminaryan, R., (2014). Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *Lancet Infect Dis.*, 14: 742-50

Veldman A., Meijs, J.A.C., Borggreve, G.J. & Heeres van der Tol, J.J. (1992). Carry over of aflatoxin from cows’ food to milk. *Anim. Prod.*, 1992:163-168

World Health Organisation (2004), *Water, Sanitation and Hygiene Links to Health. Facts and Figures.* http://www.who.int/water_sanitation_health/en/factsfigures04.pdf?ua=1 (2015-01-27)